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Expression profiling of *Cry1Aabc* gene and insect bioassay for *Helicoverpa armigera* in transgenic pigeonpea

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The major devastating insect pest of pigeonpea is *Helicoverpa armigera* which is highly susceptible to the insecticidal proteins of *Bacillus thuringiensis* (*Bt*). A codon-optimized chimeric *Cry1Aabc* gene of *Bt* driven by a constitutive promoter was introduced in pigeonpea genotype PKV Tara through embryo infection to germinating seeds and floral bud with *Agrobacterium* strain EHA 105 harboring *Cry1Aabc* gene. The present investigation of *in planta* transformation technique was undertaken to confirm and select transformants with higher level of Cry protein expression from segregating population and to study the expression of Cry endotoxin through qualitative and quantitative enzyme linked immunosorbent assay (ELISA) assay. Polymerase chain reaction (PCR) screening with gene specific markers and ELISA assay of 15 different events in T_2, T_3 and T_4 generations was carried out. Out of 220 progenies tested, 209 were positive in PCR and 208 in qualitative ELISA assay. Quantitative ELISA was performed in the selected plant on the basis of higher optical density (OD) and qualitative ELISA performed on different targeted tissue. Concentration of Cry1Ab and Cry1Ac endotoxin was recorded up to 1.347 ng/mg and 0.186 ng/mg of leaf tissue, respectively. Leaf feeding assays with neonate larvae of *H. armigera* recorded significantly higher mortality rate (89.33%) in positive plants as compared to control. In insect bioassay study it was found that expression level of Cry protein in positive plants was able to reduce the leaf damage to minimal along with mortality of larvae. The positive plants reported in present investigation had potential for providing tolerance against *H. armigera*.

Keywords: Transgenic pigeonpea, genetic transformation, *Cry1Aabc* gene, *Helicoverpa armigera*, ELISA.

Introduction

India is home place of many legumes but pigeonpea has its own importance. In India, over 150 species of insects damage pigeonpea[1], of which the pod borer, *Helicoverpa armigera* (Hubner) is the major attacking pest. Insect pest *H. armigera* has lead to series of consequences like, insecticide resistance, pest resurgence, outbreak of secondary pests, harmful residual effects, imbalances in natural ecosystem and higher production costs, which has been a concern in India and elsewhere. It is therefore requisite to develop more environment friendly approaches with minimum use of chemical pesticides to control the pest. To overcome these problems transfer of insect resistant traits is becoming a best alternative control of insect pest *H. armigera*. The biotechnological approaches such as genetic transformation using appropriate genes and DNA marker assisted selection have potential in mitigating problem of non-availability of gene in germplasm and eventually increasing the yield. Development of transgenics expressing insecticidal proteins in productive cultivars is one of the strategies followed in many crop species including pigeonpea. Use of *cry* genes from *Bacillus thuringiensis* (Bt) have been found useful in controlling the pod borer when they are introduced into crop plants[2]. It is well proven that the (Bt) crystal (Cry) protein provides important source of resistance for large group of insects of Lepidoptera family. The *Bt-Cry1Aabc* gene was constructed by taking nucleotide sequences coding for different domains from lepidopteron-specific *Bt*-toxins *viz*., The Cry proteins have three domains: a seven helix bundle (Domain I) involved in pore formation, a triple anti-parallel beta sheet domain (Domain II) for receptor binding and beta sheet sandwich (Domain III) for protease protection as well as receptor binding[3]. The present study, reports development of transgenic pigeonpea events for improved plant protection against the pod borer.

Material and Methods

Present study was carried on putative transformants of pigeonpea genotype PKV-Tara obtained through *in planta* inoculation to embryo axes of germinating

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seeds and floral bud with *Agrobacterium tumefaciens* strains EHA 105 harboring pBinAR-Cry1Aabc and the progenies were maintained in containment facilities in transgenic green house at Biotechnology Centre, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India. In present investigation 4 putative events in T_2 generation, 9 putative events in T_3 generations and 2 putative events in T_4 generation were selected.

Gene Construct

The pBinAR-Cry1Aabc binary vector (Fig. 1) was obtained through Material transfer Agreement (MTA) from National Research Center on Plant Biotechnology, New Delhi, India.

Preparation of *Agrobacterium tumefaciens* Strain EHA 105 Harbouring *Cry1 Aabc* Gene

A colony of bacteria grown for 24 hrs was taken from Petri dish and was inoculated in 1 ml of liquid Lurai Bertani (LB) medium containing 100 mg/l of kanamycin and 10 mg/l rifampicin and incubated for 24 hrs at 28°C on orbital shaker with 150 rpm. One ml culture of LB with *Agrobacterium* is transferred to 50 ml *Agrobacterium* (AB) medium and the culture is incubated at 28°C for 18 hrs on orbital shaker with 150 rpm followed by addition of 5 - 8 ml of tobacco leaf extract and further incubated for more 5 hrs at 28°C on orbital shaker with 150 rpm and used for infection[4-5].

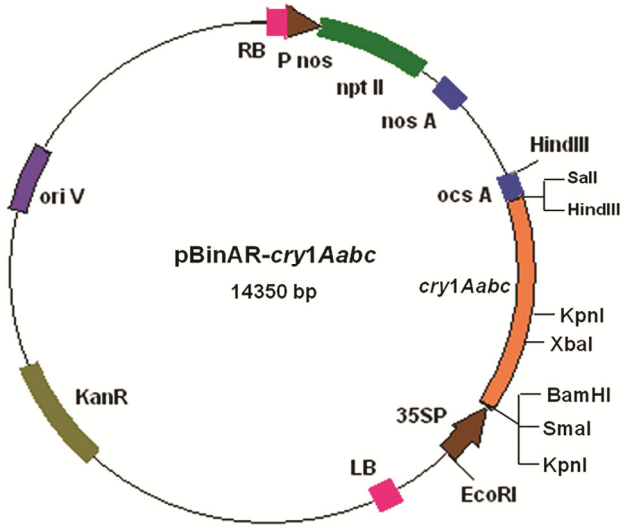

Fig. 1 — Construction of pBinAR-*Cry*1*Aabc* binary vector

Genetic Transformation through *in Planta* Technique

Seeds of pigeonpea cultivar PKV-Tara were obtained from Senior Research Scientist, Pulses Research Unit, Dr. PDKV, Akola, India.

Infection to Embryo Axes of Germinating Seed

The seeds were surface sterilized with 1% (v/v) laboline for 5 minutes, followed by washing with sterile distilled water for 5 - 6 times. The washed seeds were treated with 0.1% (w/v) mercuric chloride for 5 mins followed by rinsing with sterile distilled water for 5 - 6 times. Sterilized seeds were soaked in sterile distilled water for 48 hrs for germination. The soaked seeds were pierced at cotyledonary node point and at apical meristem of germinating embryo axis. The pierced germinating seeds were transfer in suspension of *Agrobacterium* culture harboring pBinAR-cry1Aabc and allowed for co-cultivation for different duration (30, 60 and 90 min.) with continuous agitation on orbital shaker at 50 rpm. The seeds were blot dried on filter paper and allowed to grow for 24 hrs on moist filter paper and then sown in sterile soil rite in plastic cups[4-5]. The cups were kept in transgenic green house for germination (Fig. 2).

Fig. 2 — *Agrobacterium* infection with floral dip method

Floral Dip Method

The flower bud of pigeonpea was opened before pollination and *Agrobacterium* culture was applied to the pistil with help of brush. The bud was allowed to pollinate and observed for development of pod (Fig. 3). The seeds harvested from plants of both the methods were sown in next season and screened for confirmation of any transformation.

Molecular Screening of Putative Transgenic Plants for Confirmation of *Cry1Aabc* Gene

Total genomic DNA was extracted from the young leaves by Sambrook *et al*[6]. The presence of the *Cry1Aabc* gene in the progeny of putative transgenic plants was confirmed by PCR using *Cry1Aabc* gene specific primers 5′-ttctgcccaaggtatcga-3′ (forward) and 5′-cagcctggagtgttgca-3′ (reverse) and *nptII* specific primer 5′-caatcggctgctctgatgccg-3′ (forward)

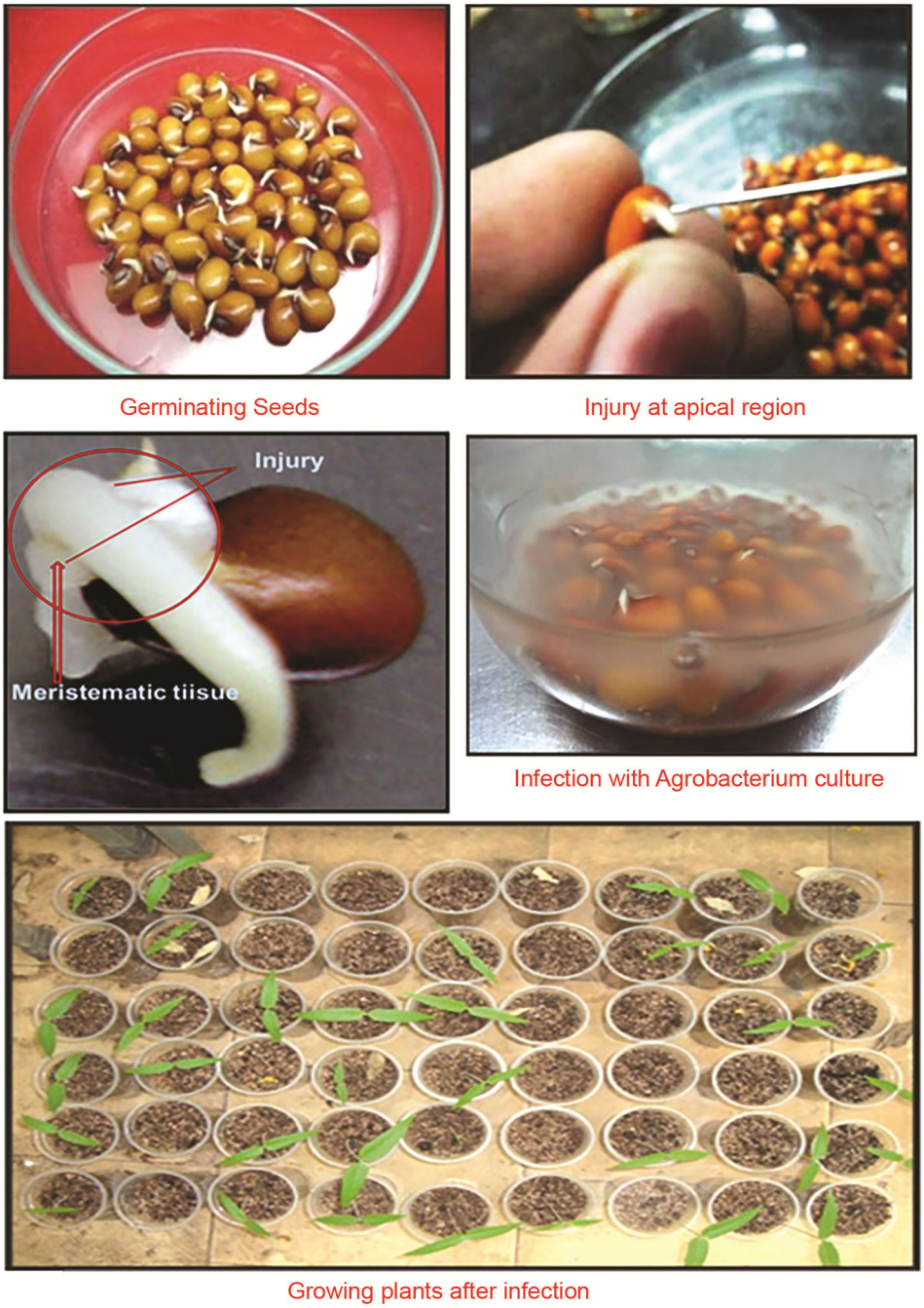

Fig. 3 — Embryo infection to germinated seed with *Agrobacterium*

and 5′-aggcgatagaaggcgatgcgc-3′ (reverse). Amplification of DNA extracted from progenies of putative plants was carried out at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, 52°C for 60 s for annealing and 72°C for 5 min, with a final extension at 72°C for 10 min. PCR products was resolved on 1.5% ethidium bromide (EtBr) stained agarose gel in 1X TBE buffer. Gel images were documented using gel documentation system (BioRad Gel Doc, USA).

Detection of Cry1Ac/Ab Protein in Transgenic Pigeonpea Plants

ELISA kit for Cry1Ac/Ab (Amar Diagnostic) was used for estimation of Cry1Ac and Cry1Ab protein in progenies of transgenic pigeonpea plants. The Cry1Aabc protein was detected by sandwich-ELISA using cell-free extracts of young leaf sample according to manufacturer's instructions. All PCR-positive transgenic pigeonpea progenies of 15 different events was analyzed by ELISA and used for further analysis.

Qualitative ELISA

Leaf samples were ground by rotating the pestle against the sides of the tube with twisting motions until the tissue were well grounded. The grounded samples were incubated at room temperature for 20 mins. Fifty µl supernatant was extracted and transferred to individual wells of microtiter plate. Fifty µl of positive and negative control were added in respective wells. One hundred µl of Cry1Ab/Ac-enzyme conjugate was added in each well, followed by incubation for 40 min at room temperature. Wells were washed four times with wash solution. To each well, 100 µl of substrate was added and plate was incubated for 20 min at room temperature. To each well 100 µl of stop solution was added and finally reading was taken at 450 nm.

Quantitative ELISA

Sample Extraction (Pigeonpea Leaf Tissue) and Sample Dilution - Prior to Assay

Six leaf punch samples were taken by snapping the tube cap of the disposable tissue extractor down on the leaf. The pestle was inserted into the tube and the tissue was grinded by rotating the pestle against the sides of the tube with twisting motions. Further, dilution (1:11) was carried out and 50 µl sample extract was added and mixed.

Procedure

One hundred µl of negative control, 100 µl of each calibrator and 100 µl of each sample extract were added to their respective wells. Thoroughly, the contents of the wells were mixed and incubated at ambient temperature for 15 min. After incubation 100 µl of *Cry1Ab/Ac*-enzyme conjugate was added to each well and incubated at ambient temperature for 1 hr. After incubation, wells were washed for 4 times with wash buffer using a microtiter plate or strip washer. One hundred µl of substrate was added to each well and incubated for 30 mins at room temperature. One hundred µl of stop solution was added to each well and mixed thoroughly and reading was taken at 450 nm in ELISA reader. The optical density (OD) value of each calibrator and corresponding concentrations of Cry1Ac and Cry1Ab were used to prepare the standard curve. The concentration of each sample was determined by finding its OD value and the corresponding concentration level in graph and these results were multiplied with all dilution factors incurred during extraction and the results were reported as nanogram toxin per gram of leaf tissue.

Insect Bioassays

After confirmation of *Cry1Aabc* gene expression in progenies of selected events, insect bioassay with pod borer (*H. armigera*) was performed. The bioassays was performed in the laboratory at temperature 27 ± 2ºC and 65 - 75 relative humidity (RH) with a photoperiod 12:12 (L:D hrs). The larvae were reared on artificial diet and 5 days old larvae were used for detached leaf bioassay. The third fully expanded leaf from the top of the pigeonpea plant was selected for the bioassay. The experiment was carried out in 3% agar containing bottles. The solidified agar was used as substratum for holding pigeon pea leaf in slanting manner inside the bottles. Ten neonates *H. armigera* larvae were released to on the pigeonpea leaves. The observations were recorded after 5th day of released larvae in bottles. Weight of larvae was recorded after 5th day, leaf area damaged was estimated by scale (in %) and mortality rate was calculated[7].

Results and Discussion

Molecular Analysis of Transgenic Pigeonpea

Integration of *Cry1Aabc* gene among the progeny of different generations (T_2, T_3 and T_4) of putative transformants of pigeonpea was confirmed through PCR amplification with gene specific marker i.e. *Cry1Aabc* (1000 bp) and *nptll* (750 bp). The amplification products resolved on 0.8% agarose gel were observed for amplicon of 1000 bp and 750 bp (Fig. 4 & 5). In all progeny of 15 different events in

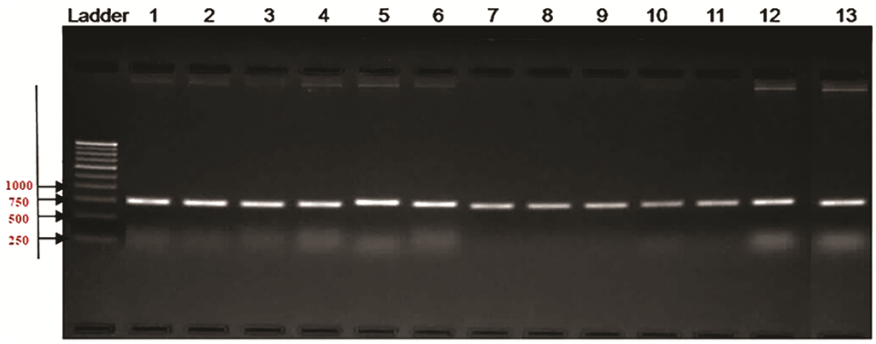

Fig. 4 — PCR result of *nptII*. Ladder- 1Kb, 1- Positive, 2-Negative

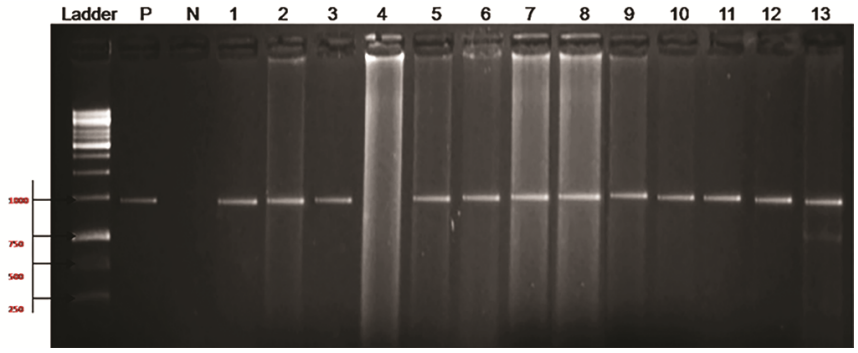

Fig. 5 — PCR result of *Cry1Aabc*. Ladder- 1Kb, P- Positive, N-Negative

different generation was tested. Out of 15, progeny of 11 events was 100% positive in PCR testing. In all 209 progenies were found to be positive out of 220 progenies tested by PCR by gene specific primers (Table 1). The PCR positive progenies were subjected for qualitative ELISA for confirming the expression of Cry1Ac and Cry1Ab protein by the positive plants.

Detection of Cry1Ac/Ab Protein

Qualitative ELISA Assay

The sandwich ELISA is an accurate and sensitive technique used for detection and quantification of Cry protein in the transgenic plants. In present study, the expression of Cry1Ac in plants of different generation was detected by ELISA using cell free extract. The optical density (OD) of the sample was monitored at 450 nm using the microtiter plate reader of which data is presented in Table 2. Qualitative results of the assay were visualized with color development (Fig. 6). Out of 220 progenies tested 208 were positive in qualitative ELISA assay. The progenies which had recorded highest O.D were in T_3 generation of floral dip and embryo infection. The data recorded

Table 1 — Molecular analysis of T2, T3 and T4 generation of transgenic pigeonpea.

Plant no./ Event no.	Generation	No. of plant tested	No. of PCR negative plants	No. of PCR positive plants
Floral dip method				
1	T_2	07	0	07
2	T_2	10	0	10
3	T_3	30	4	26
4	T_3	30	2	28
Embroy infection method				
5	T_2	15	2	13
6	T_2	14	0	14
7	T_3	19	0	19
8	T_3	20	0	20
9	T_3	19	0	19
10	T_3	19	0	19
11	T_3	12	0	12
12	T_3	09	0	09
13	T_3	12	3	09
14	T_4	02	0	02
15	T_4	02	0	02
Control			0	
Total		220	11	209

Table 2 — Qualitative assessment of plants with Cry1Ac/Ab ELISA kit.

Plant no. / Event no.	Generation	Total no of seed sown	Total number of plant subjected to ELISA	ELISA positive	ELISA negative
			Floral dip method		
1	T_2	10	7	7	0
2	T_2	10	10	10	0
3	T_3	30	30	26	4
4	T_3	30	30	28	2
			Embryo infection		
5	T_2	15	15	13	2
6	T_2	15	14	14	0
7	T_3	20	19	19	0
8	T_3	20	20	20	0
9	T_3	20	19	19	0
10	T_3	20	19	19	0
11	T_3	15	12	12	0
12	T_3	10	9	9	0
13	T_3	15	12	8	4
14	T_4	5	2	2	0
15	T_4	5	2	2	0
		Total	220	208	12

Cut off value=Negative absorbance + 0.1 Positive= above cut off value
Cut off value= 0.1+0.1= 0.2 Negative= bellow cut of value

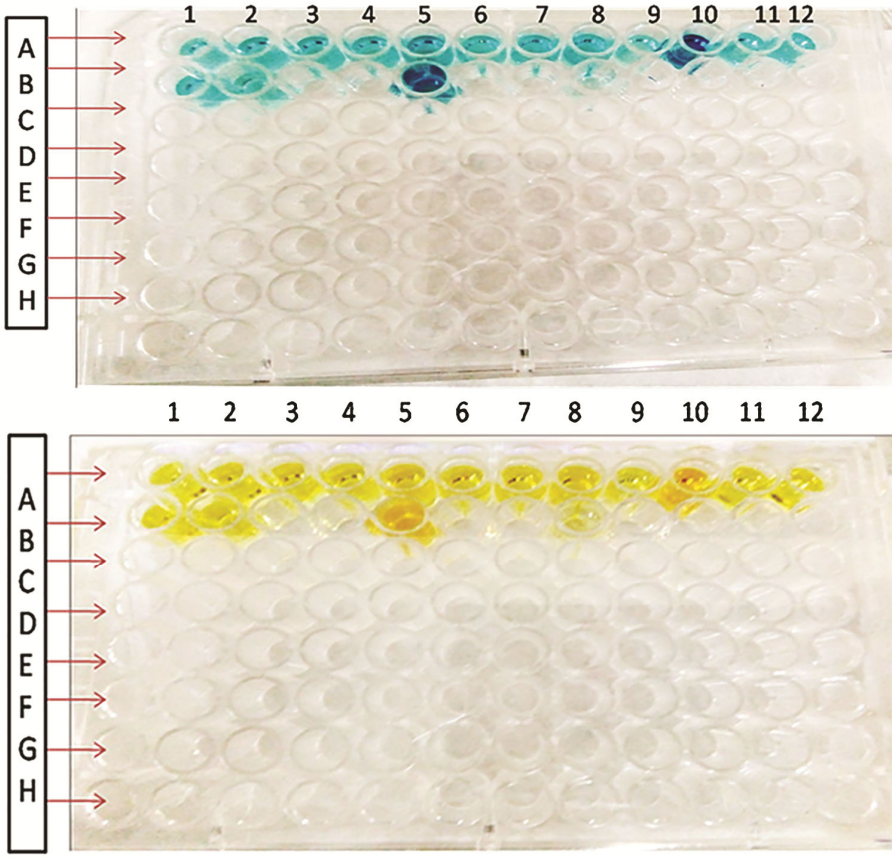

Fig. 6 — Qualitative ELISA assay of progeny of plant no.3
Well A1 to B2: Progeny of event no.3 (T_3 generation) of floral dip and Well B3: Blank well B4: Negative well B5: Positive well.

in qualitative ELISA was used for selecting the plants for quantitative assay. The two plants from each set of progenies showing OD above 0.5 were selected.

Quantitative ELISA Assay

Envirologix quantiplate ELISA Kit for Cry1Ac/ Cry1Ab was used for quantitative assessment of Cry1Ab and Cry1Ac endotoxin in progenies of putative transgenic pigeonpea plants. Such ELISA kit had also been used to estimate Cry1Ac toxin in tobacco and cotton transgenic plants. The expression of Cry1Ac toxin in transgenic plants was detected by ELISA using cell free extract. Expression levels of Cry protein in transgenic pigeonpea plants were quantified on the basis of linear standard curve plotted with positive calibrator.

The concentration of Cry1Ab endotoxin ranged between 0.125 ng/mg of leaf tissue to 1.347 ng/mg of leaf tissue and the concentration of Cry1Ac endotoxin ranged between 0.134 ng/mg of leaf tissues to 0.186 ng/mg of leaf tissue for the plants under study. Out of plant progenies tested with ELISA, floral dip plant of T_4 generation, had recorded highest concentration of Cry1Ab and Cry1Ac endotoxin i.e. 1.347 ng/mg and 0.186 ng/mg respectively (Fig. 7 & Table 3).

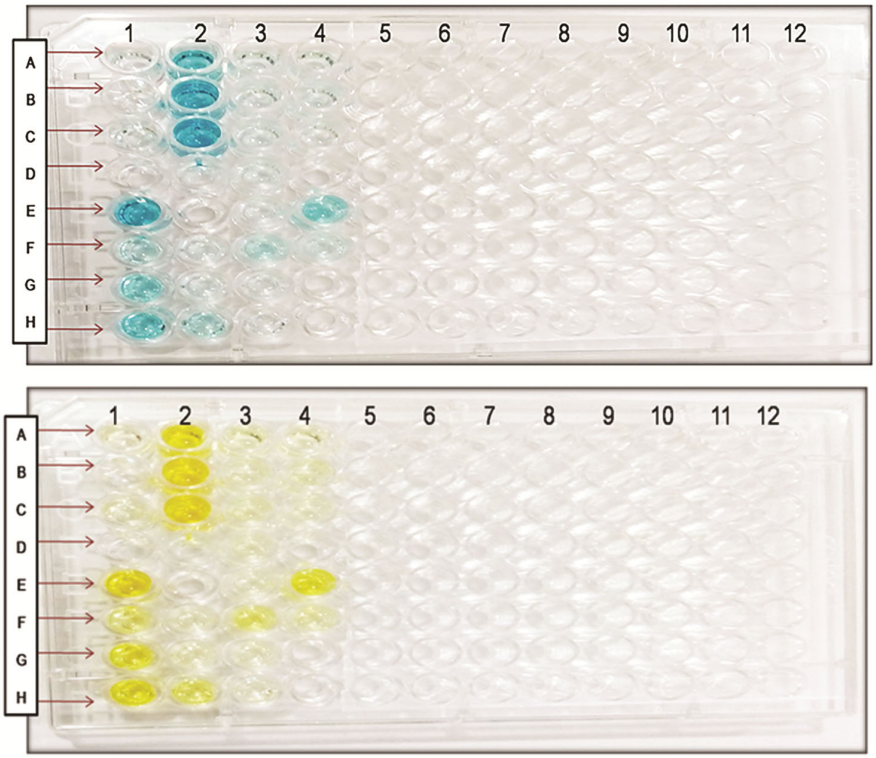

Fig. 7 — Quantitative ELISA assay of selected 7 plants.
Well E2, F2, G2 to E4: seven samples (T_3T_4, & T_5 generation); Well F1, G1, H1, A2, B2 & C2: Calibrator well A1: Blank and Well C1: Negative well E1: Positive

Table 3 — Analysis of advance generation by quantitative ELISA

Sr. No.	Event no./ plant no.	Cry1Ab endotoxin Concentatrion (ng/mg of leaf tissue) (*diluted sample)	Cry1Ac endotoxin Concentration (ng/mg of leaf tissue)
1	2	0.209	0.155
2*	3	1.347	0.186
3	5	0.153	0.141
4	8	0.196	0.151
5	9	0.125	0.134
6	10	0.134	0.137
7	11	0.130	0.136

(*Dilution factor= concentration of sample x 11 x 50 / 1000) (**dilution factor x 3)

Das *et al*[8] reported *Cry1Aabc* derived from three different *Bt* genes (*Cry1Aa*, *Cry1Ab* and *Cry1Ac*) for improving the level of resistance. Among the lepidopteran specific δ-endotoxins of *Bt*, Cry1A class is the most effective toxin against pod borer *H. Armigera*[9]. Attempts to improve the level of toxicity of Cry1Ac by domain shuffling have led to the finding that a chimera of Cry1Aa, Cry1Ab and Cry1Ac was more effective than Cry1Ac. Quantitative ELISA have been applied for selection of transgenic events[10-11]. Gopalswami *et al*[12] detected the expression of Cry1Ab protein by ELISA which found to be 0.07-1.26 ng/mg. They had shown expression of Cry1Ab protein in the range of 0.07-1.26 ng/mg whereas the expression for Cry1Ab protein reported in present investigation comparatively high.

Insect Bioassay

Bioassay was carried out to evaluate the potential of transformed pigeonpea plants for control of *Helicoverpa* larvae. Seven putative transformants of pigeonpea were subjected to insect (*H. armigera*) bioassay. Observation were recorded on average larval weight in milligram, percent weight reduction as compared to the control, leaf area damage estimated by scale 1 - 10 and number of larvae survives. The data revealed that the positive plants had expressed cry protein in sufficient concentration to reduce the weight of larvae and leaf damage as shown in Table 4. The results showed a clear difference between transgenic and control plants in terms of leaf consumption and larval mortality. There was extensive feeding of plant tissues more than the larvae in control plant as compared to transgenic plants. All positive plant resulted in larval weight reduction. The percent weight reductions in transgenic plants were between 90-69%. Plants of T_2 generation showed highest weight reduction up to 90% (Table 4).

It was observed that all positive plants showed lesser leaf damaged as compare to the control i.e. PKV Tara after the interval of 5 days. Thus, the results revealed that the levels of *Cry* protein expressed in positive plants are able to reduce the weight of larvae and reduce the leaf damage. Thus, it was concluded from above results that positive plants selected for bioassay study having potential for providing tolerance against *H. armigera.*

The expression of chimeric *Bt* gene, *Cry1Aabc* in transgenic pigeonpea (*cv*. Asha) for gram pod borer (*H. armigera* Hubner) was reported (Das *et al*. 2016). In the study two pigeonpea lines IPCc1 and IPCc2 were subjected to insect bioassay using 5 and 7 days old larvae and young trifoliate leaves and pods, respectively. Wild type (non-transformed) cv. Asha was used as control. Larval mortality between the controls did not differ significantly. But in present investigation there was significant difference between the larval mortality and control (Fig. 8). The present investigation established that the chimeric Cry1Aabc protein is effective against *H. armigera* larvae in insect bioassays carried across generations. Molecular characterization and insect bioassays confirmed seven superior events in pigeonpea. These seven events will be further submitted for Southern blot hybridization to detect the copy number of *Cry1Aabc* gene and the best event will be further selected for incorporation in breeding program of pigeonpea.

Table 4 — Observations recorded for insect bioassay by detached leaf method.

Plant no/ Event no.	Average larval wt (gm)	% Weight reduction over control	% Mortality rate	Leaf area damage rating (%) after five days internal
Control	0.024	0.0	13.33	79.00(62.71*)
T2-2	0.003	90.079	66.00	38.33(38.14*)
T2-3	0.007	68.91	89.33	61.67(51.90*)
T2-5	0.058	90.21	77.33	46.67(43.07*)
T2-8	0.051	89.93	76.66	46.47(43.06*)
T3-9	0.003	85.54	87.33	11.67(19.29*)
T4-10	0.004	84.12	81.00	15.00(22.28*)
T4-11	0.064	95.92	87.33	75.00(60.05*)
C.D.	0.005	12.5	9.779	9.171
SE(m)	0.002	4.14	3.234	3.033
SE(d)	0.002	5.86	4.573	4.289
C.V.	10.076	9.49	7.748	12.342

* figured data has performed angular transformed values.

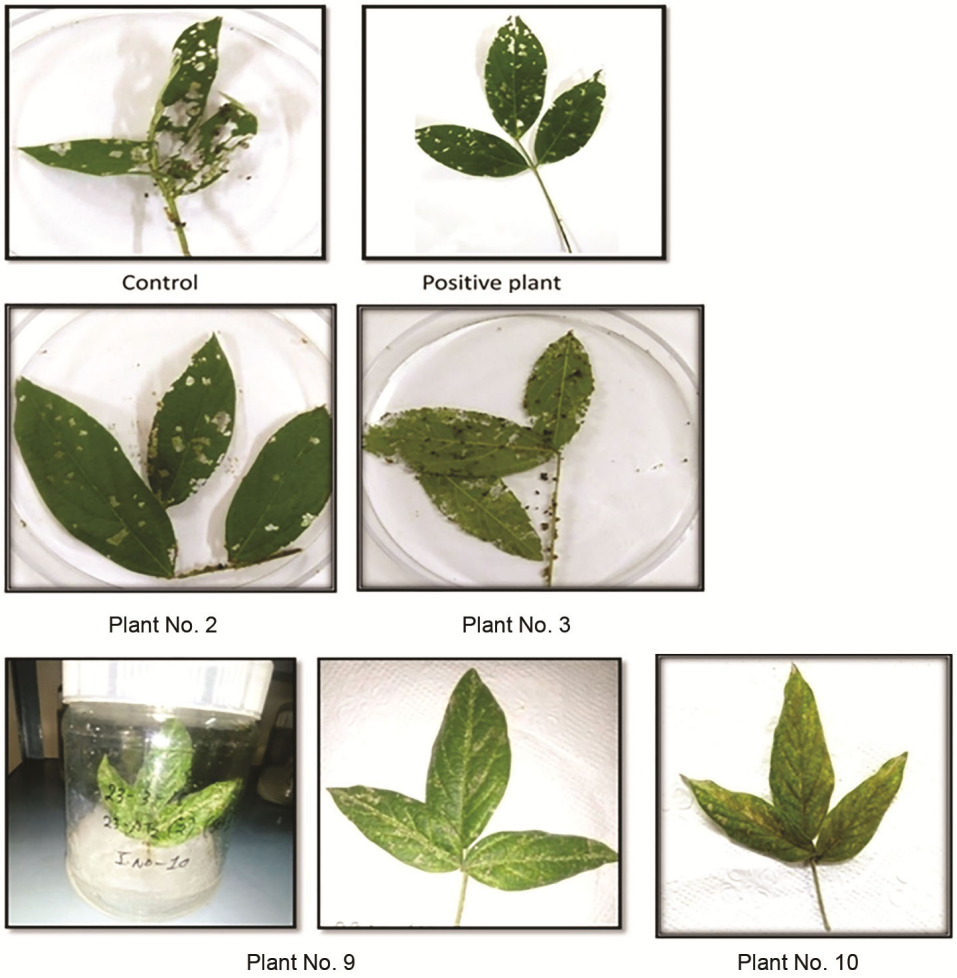

Fig. 8 — Leaf area damaged by *Helicoverpa armigera* during insect bioassay

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